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Single-tier testing with the C6 peptide ELISA kit compared with two-tier testing for Lyme disease☆,☆☆,★

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ABSTRACT

For the diagnosis of Lyme disease, the 2-tier serologic testing protocol for Lyme disease has a number of shortcomings including low sensitivity in early disease; increased cost, time, and labor; and subjectivity in the interpretation of immunoblots. In this study, the diagnostic accuracy of a single-tier commercial C6 ELISA kit was compared with 2-tier testing. The results showed that the C6 ELISA was significantly more sensitive than 2-tier testing with sensitivities of 66.5% (95% confidence interval [CI] 61.7–71.1) and 35.2% (95% CI 30.6–40.1), respectively ($P < 0.001$) in 403 sera from patients with erythema migrans. The C6 ELISA had sensitivity statistically comparable to 2-tier testing in sera from Lyme disease patients with early neurologic manifestations (88.6% versus 77.3%, $P = 0.13$) or arthritis (98.3% versus 95.6%, $P = 0.38$). The specificities of C6 ELISA and 2-tier testing in over 2200 blood donors, patients with other conditions, and Lyme disease vaccine recipients were found to be 98.9% and 99.5%, respectively ($P < 0.05$, 95% CI surrounding the 0.6 percentage point difference of 0.04 to 1.15). In conclusion, using a reference standard of 2-tier testing, the C6 ELISA as a single-step serodiagnostic test provided increased sensitivity in early Lyme disease with comparable sensitivity in later manifestations of Lyme disease. The C6 ELISA had slightly decreased specificity. Future studies should evaluate the performance of the C6 ELISA compared with 2-tier testing in routine clinical practice.

1. Introduction

Lyme disease, a tick-borne spirochetosis, has been recognized as the most frequent vector-borne disease in the United States since its identification over 2 decades ago (Bacon et al., 2008; Steere et al., 1983). The mainstay of laboratory diagnosis is detection of antibody to *Borrelia burgdorferi*, the etiologic agent (Aguero-Rosenfeld et al., 1993; Aguero-Rosenfeld et al., 2005; Craft et al., 1986; Grodzicki and Steere, 1988; Wormser et al., 2006). To improve the specificity of serologic testing for Lyme disease, a 2-tier approach was recommended in 1995 (CDC, 1995). Accordingly, a serum sample yielding a positive or indeterminate result in the first-tier assay (typically an ELISA) is retested by separate IgM and IgG immunoblots usually based on *B. burgdorferi* whole cell sonicate (WCS). The immunoblot is the second tier assay and must be positive for the serum to be considered seropositive.

The public health service recommendations in support of 2-tier testing (CDC, 1995) provide for the development of alternatives to 1 or both steps provided that equal or better performance is demonstrated by such alternative methods (Proceedings of the Second National Conference on Serologic Diagnosis of Lyme Disease, 1995). In principle, an ELISA of sufficiently high specificity and sensitivity would provide diagnostic information similar to existing 2-tier testing. A variety of recombinant and synthetic antigens have been evaluated in previous studies for use in serodiagnosis of Lyme

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disease (Bacon et al., 2003; Branda et al., 2010; Cinco and Murgia, 2006; Embers et al., 2007a, 2007b; Gomes-Solecki et al., 2007; Gottner et al., 2004; Hauser and Wilske, 1997; Heikkila et al., 2002, 2003; Jobe et al., 2008; Lawrenz et al., 1999; Liang et al., 1999a, 1999b; Magnarelli et al., 2000, 2002; Mathiesen et al., 1998; Mogilyansky et al., 2004; Padula et al., 1994; Panelius et al., 2001; Peltomaa et al., 2004; Rasiah et al., 1994; Sillanpaa et al., 2007; Skarpaas et al., 2007; Smismans et al., 2006; Steere et al., 2008; Tjernberg et al., 2007, 2008; Wormser et al., 2008a). In particular, the VlsE protein and the highly conserved 25-amino acid peptide ("C6 peptide") derived from the sixth invariant region of this protein have been shown to be both sensitive and specific antigens in the ELISA (Bacon et al., 2003; Cinco and Murgia, 2006; Embers et al., 2007a, 2007b; Gomes-Solecki et al., 2007; Gottner et al., 2004; Heikkila et al., 2003; Lawrenz et al., 1999; Liang et al., 1999a, 1999b; Magnarelli et al., 2002; Mogilyansky et al., 2004; Peltomaa et al., 2004; Sillanpaa et al., 2007; Skarpaas et al., 2007; Smismans et al., 2006; Steere et al., 2008; Tjernberg et al., 2007, 2008; Wormser et al., 2008a) and immunoblot formats (Branda et al., 2010).

The aim of the present study was to determine whether the C6 ELISA by itself is a suitable alternative to 2-tier testing by evaluating the comparative diagnostic accuracy in over 550 sera from well-characterized Lyme disease patients and in more than 2200 control sera.

2. Methods

2.1. Serologic assays

The C6 Lyme ELISA kit (Immunetics, Boston, MA, USA), a test kit approved by the Food and Drug Administration (FDA) for use as a first-tier assay, was modified for this study. Kits provided by the manufacturer for this study incorporated a simplified and comparable cut-off formula that was based on a negative calibrator serum. This modification yielded sensitivity and specificity statistically equivalent to what had been demonstrated with the original cut-off formula. Reproducibility testing of the C6 ELISA kit using the simplified cut-off yielded intra-assay and interassay coefficients of variation (CV) of 10% and 11.6%, respectively. Intra-assay and interassay CVs for the kit with the original cut-off were 9.5% and 14.3%, respectively. In all other respects, the modified and original kits were the same.

The C6 peptide used as antigen in the kit is derived from the *B. burgdorferi* B31 strain sequence, which differs from the originally described IP90 sequence (Liang et al., 1999b) by 4 amino acids. The kit is formatted as an indirect ELISA in which both IgG and IgM antibodies to C6 peptide are detected by an enzyme conjugate. The C6 ELISA testing was performed at New York Medical College, the Centers for Disease Control and Prevention (CDC), and at Immunetics. Two-tier serology was performed using IgG/IgM ELISA kits from Wampole Laboratories (Princeton, NJ, USA) (at New York Medical College and Immunetics) or IgG/IgM Vidas II Lyme Screening kits from bioMerieux (Durham, NC, USA) (at the CDC), followed by Lyme IgG and IgM immunoblot kits from MarDx/Trinity Biotech (Carlsbad, CA, USA). The 2-tier ELISA and immunoblot kits were based on a *B. burgdorferi* WCS antigen and were also FDA approved for in vitro diagnostic use. An alternative 2-tier result was calculated based on the C6 ELISA result as the first step, combined with the same IgG and IgM immunoblot results as above. For the majority of the Lyme disease sera tested, the WCS ELISA–2-tier serologic testing was performed as part of the current study. However, for 156 sera from one of the panels for which the volume of serum was limited, we instead used the WCS ELISA–2-tier serologic test results that were obtained in 2002 as part of another study (Bacon et al., 2003); these tests had also been performed using the Vidas II Lyme Screening kit and the MarDx/Trinity Biotech kits.

2.2. Lyme patient sera

The patient sera selected for this study were chosen to represent a broad range of clinical manifestations of Lyme disease (Table 1) from well-characterized patients. The sera comprised multiple reference panels from different sources that had been previously collected and frozen at −80 °C. The 569 Lyme disease sera studied were obtained from 528 patients. Erythema migrans was defined based on clinical diagnosis alone for 172 sera and on clinical diagnosis in conjunction with microbiologic confirmation of *B. burgdorferi* infection by either culture or polymerase chain reaction (PCR) for 231 sera. The test results for some of these sera were previously reported (Wormser et al., 2008a, 2008b).

Early neurologic Lyme disease was defined based on the presence of objective clinical (e.g., facial nerve palsy) and/or laboratory findings (e.g., cerebrospinal fluid lymphocytic pleocytosis) in association with concomitant/recent erythema migrans for 39 sera; for the other 5 sera, evidence for *B. burgdorferi* infection was based on prior seropositivity by at least a positive WCS ELISA on an acute- or convalescent-phase serum specimen. Late neurologic Lyme disease was defined based on the presence of a compatible objective clinical finding (e.g., encephalopathy, polyneuropathy, or encephalomyelitis) in association with serologic evidence of borrelial infection demonstrated by at least a positive WCS ELISA. Acute erythema migrans or neurologic Lyme disease sera were collected prior to or on the date of first antibiotic treatment; convalescent sera were collected after the completion of antibiotic treatment.

Lyme arthritis was defined as the presence of joint swelling that was clinically compatible with Lyme arthritis in conjunction with

Table 1
Sera selected for evaluation.

Panel	*n*	Culture or PCR positive[b]	Days post onset of symptoms							
			0–7	8–14	15–21	22–30	31–60	61–90	> 90	NA[c]
All erythema migrans	403	231	160	65	42	34	76	13	10	3
Single[a]	133	131	65	30	22	7	5	1	3	0
Multiple[a]	58	57	27	18	7	3	2	1	0	0
Acute	298	209	159	65	37	19	14	1	0	3
Convalescent	105	22	1	0	5	15	62	12	10	0
Early/acute neurologic Lyme disease	20	4	3	2	3	2	1	5	4	0
Early/convalescent neurologic Lyme disease	24	5	4	3	3	2	4	2	6	0
Late neurologic Lyme disease	8	0	0	0	0	0	0	0	6	2
Arthritis	114	10	0	0	1	0	6	4	50	53
Total	569	250	167	70	49	38	87	24	76	58

[a] Erythema migrans not characterized as single or multiple for 212 sera.
[b] Not all sera were from patients whose blood or skin lesion was tested by culture or PCR.
[c] Time after onset data not available.

Table 2
Reactivity in blood donor populations.

Method	Number [percent] of negative[a] (95% CI)[b]		
	Nonendemic	Endemic	Total
C6 ELISA	509 [99.2%] (98.0–99.8%)	1311 [98.6%][c] (97.9–99.2%)	1820 [98.8%] (98.2–99.3%)
Two-tier (WCS ELISA/WB)[d]	512 [99.8%] (98.9–100%)	1321 [99.4%][c] (98.8–99.7%)	1833 [99.5%] (99.1–99.8%)
Two-tier (C6 ELISA/WB)[e]	513 [100%] (99.3–100%)	1320 [99.3%] (98.7–99.7%)	1833 [99.5%] (99.1–99.8%)
WCS ELISA	492 [95.9%] (93.8–97.4%)	1283 [96.5%] (95.4–97.5%)	1775 [96.4%] (95.4–97.2%)
n (total no. of donors)	513	1329	1842
P[f]	0.38	0.006	0.002
P[g]	0.13	0.004	<0.001

WCS = Whole cell sonicate.
[a] % Negative calculated as (total no. of samples − total no. of reactive samples)/total no. of samples.
[b] Calculated by the exact method.
[c] One donor serum which was positive by both C6 ELISA and IgG immunoblot, but negative in WCS ELISA, was categorized as 2-tier negative.
[d] Two-tier testing with WCS ELISA as the first step and IgG + IgM Western blot as the second step.
[e] Two-tier testing with C6 ELISA as the first step and IgG + IgM Western blot as the second step.
[f] For the comparison of C6 ELISA with WCS ELISA-based 2-tier testing.
[g] For the comparison of C6 ELISA with C6 ELISA-based 2-tier testing.

serologic evidence of borrelial infection demonstrated by at least a positive WCS ELISA.

Serum samples which had been previously found to be seronegative by an ELISA and which had been obtained from patients with erythema migrans in whom the diagnosis was confirmed by culture or PCR ($n = 58$) were purposely included in this study to challenge the sensitivity of the methods under evaluation.

2.3. Controls

Serum samples were obtained from healthy blood donors from endemic (US Northeast) and nonendemic regions for Lyme disease (US Southeast and Southwest). In addition, other control sera were tested from patients with other diseases or conditions and from recipients of the LymeRx® vaccine (SmithKline Beecham, Philadelphia, PA, USA).

The protocol was approved by the Institutional Review Board of New York Medical College. Serum panels were either obtained from volunteers who gave written informed consent to have their serum used to improve diagnostic tests for Lyme disease or came from anonymous, preexisting reference samples that had previously been unlinked from the serum donor.

2.4. Data and statistical analysis

Sensitivity was calculated as percent positive in the Lyme disease populations, while specificity was calculated as percent negative in the control sera. Comparisons were made using the Fisher exact test. Where appropriate, McNemar's test was used to account for the paired measurements on samples. *P* values were 2-tailed and a value of <0.05 was considered significant. All analyses were conducted using Stata (version 11.2, StataCorp, College Station, TX, USA).

3. Results

3.1. Specificity

Serum samples were obtained from healthy blood donors from endemic ($n = 1329$) (US Northeast) and nonendemic ($n = 513$) regions for Lyme disease (US Southeast and Southwest) (Table 2). In addition, 399 other control sera were tested including sera from individuals with the conditions listed in Table 3 and sera from recipients of the LymeRx® vaccine ($n = 33$).

Specificities of the C6 ELISA and WCS ELISA-based 2-tier testing in a population of healthy blood donors from regions of the USA nonendemic for Lyme disease were both above 99%, but the specificity of 2-tier testing exceeded that of the C6 ELISA by 0.6 percentage points (95% CI surrounding the 0.6 percentage point difference of 0 to 2.0, $P = 0.38$) (Table 2). Among donors from regions endemic for Lyme disease, the specificities of both the C6 ELISA and 2-tier testing were approximately 0.5 percentage points lower than among donors from nonendemic areas (98.6% versus 99.4%, respectively; $P = 0.006$).

Table 3
Reactivity with sera from patients with other diseases or that may contain interfering antibodies or substances.

Disease condition	*n*	No. of positive sera			
		C6 ELISA Positive or indeterminate	Two-tier (WCS ELISA)[c] positive	Two-Tier (C6 ELISA)[d] positive	WCS ELISA Positive or indeterminate
Helicobacter pylori	20	0	1	0	3
Mycoplasma pneumoniae	38	0	0	0	6
Cytomegalovirus	20	0	0	0	4
Epstein–Barr virus	20	0	0	0	3
Human immunodeficiency virus	20	0	0	0	2
Hepatitis A	25	0	0	0	1
Hepatitis B	23	0	0	0	4
Hepatitis C	15	0	0	0	5
Influenza vaccinated	25	0	0	0	0
Antinuclear antibody +	20	0	0	0	1
Rapid plasma reagin +[a]	20	1	1	1	3
Lipemic	20	0	0	0	4
Icteric	20	0	0	0	0
Hemolyzed[a]	20	1	1	1	1
Systemic lupus erythematosus	20	0	0	0	0
Rheumatoid arthritis	20	0	0	0	1
Rheumatoid factor +	20	0	0	0	1
Total	366	2	3	2	39
Overall no. [%] of negative[b] (95% CI)		364 [99.5%] (98.0–99.9%)	363 [99.2%] (97.6–99.8%)	364 [99.5%] (98.0–99.9%)	327 [89.3%] (85.7–92.3%)

[a] The same serum sample was positive in all assays.
[b] % Negative calculated as (total no. of samples − total no. of reactive samples)/total no. of samples.
[c] Two-tier testing with WCS ELISA as the first step and IgG + IgM Western blot as the second step.
[d] Two-tier testing with C6 ELISA as the first step and IgG + IgM Western blot as the second step.

Among the entire blood donor population of 1842 individuals, 2-tier testing was associated with a significantly higher specificity of 99.5% compared with 98.8% for the C6 ELISA ($P = 0.002$, 95% CI surrounding the 0.7 percentage point difference of 0.11 to 1.36).

Testing 366 sera from individuals with 14 other disease conditions, or with several common potential interferences, yielded a specificity for the C6 ELISA of 99.5% (95% CI 98.0–99.9) compared with 99.2% (95% CI 97.6–99.8) for WCS ELISA-based 2-tier testing (Table 3) ($P = 1.0$). The 2 sera that were positive by C6 ELISA in this group were also positive by 2-tier testing. Of the 33 LymeRx® vaccinee sera, 1 was C6 ELISA positive; none was 2-tier positive, although 29 were positive and 1 was in the indeterminate range on WCS ELISA.

Overall, the specificities of C6 ELISA and WCS ELISA-based 2-tier testing in the 2241 control sera evaluated were 98.9% and 99.5%, respectively ($P < 0.05$; 95% CI surrounding the 0.6 percentage point difference of 0.04 to 1.15). Among the 25 sera that were reactive by the C6 ELISA, 9 (36.0%) tested positive by the 2-tier method. Conversely, among the 12 sera that were reactive by 2-tier testing, 9 (75%) were positive by the C6 ELISA. By comparison, the specificity of the first-tier WCS ELISAs was significantly less than that of the C6 ELISA for the 4 groups of controls (95.9% for WCS ELISA versus 99.2% for C6 ELISA in nonendemic blood donors, $P < 0.001$; 96.5% for WCS ELISA versus 98.6% for C6 ELISA in endemic blood donors, $P < 0.001$; 89.3% for WCS ELISA versus 99.5% for C6 ELISA in other diseases or interfering conditions, $P < 0.001$; and 9.1% for WCS ELISA versus 97.0% for C6 ELISA in LymeRx® vaccine recipients, $P < 0.001$) (Tables 2 and 3).

Substitution of the C6 ELISA for the WCS ELISA in the 2-tier testing yielded essentially identical results for specificity in each of the control groups (Tables 2 and 3).

3.2. Sensitivity

3.2.1. Sensitivity versus disease manifestation

Sample demographics are shown in Tables 1 and 4. Patients who had both erythema migrans and an extracutaneous manifestation were tabulated only under the category of the extracutaneous manifestation in this analysis. The overall sensitivities of the C6 ELISA and of WCS ELISA-based 2-tier testing were 75.0% (95% CI 71.3–78.5) and 51.5% (95% CI 47.3–55.7), respectively, in the group of 569 Lyme disease sera previously collected from 528 patients (Table 4) ($P < 0.001$). Sera were selected for this study to represent a broad range of clinical manifestations of Lyme disease. The elimination of the 157 sera from patients who had previously been treated with antimicrobials did not significantly ($P = 0.5$) alter these values (overall sensitivity for C6 ELISA = 73.1%, 95% CI 68.5–77.3; for WCS ELISA-based 2 tier testing = 53.9%, 95% CI 48.9–58.8). The higher sensitivity of the C6 ELISA was entirely due to the insensitivity of the second-tier immunoblot test, primarily during early disease; indeed, the sensitivity of the first-tier WCS ELISA exceeded that of the C6 ELISA (81.7% versus 75.0% for all Lyme sera combined, $P < 0.001$).

On acute-phase samples, the difference in sensitivity between the C6 ELISA and WCS ELISA-based 2-tier testing was most evident in the group of sera from patients presenting with a single erythema migrans skin lesion, in which the sensitivity of the C6 ELISA was more than twice that of 2-tier testing (57.9% versus 27.1%, $P < 0.001$) (Table 4). In sera from patients with multiple erythema migrans skin lesions, the sensitivity of both methods rose substantially, but the C6 ELISA was 41% more sensitive than 2-tier testing in this subgroup ($P < 0.001$) (Table 4). Seropositivity on acute-phase serum samples of culture or PCR-confirmed cases of erythema migrans was similar to that of clinically defined cases for both the C6 ELISA (135/209 [64.6%, 95% CI 57.7–71.1] versus 56/89 [62.9%, 95% CI 52.0–72.9]) and for 2-tier testing (80/209 [38.3%, 95% CI 31.7–45.2] versus 34/89 [38.2%, 95% CI 28.1–49.1]).

In patients with early neurologic Lyme disease including facial palsy, meningitis, and radicular neuropathy, a total of 20 sera obtained during the acute phase before antibiotic treatment and 24 obtained during the convalescent phase after antibiotic therapy was initiated were evaluated in this study. The convalescent-phase samples were obtained from patients separate from those who provided the acute-phase serum samples. The sensitivity of the C6 ELISA was comparable to that of WCS ELISA-based 2-tier testing in these groups (90.0% versus 80.0% [$P = 0.50$] in the acute-phase serum samples and 87.5% versus 75.0% [$P = 0.38$] in the convalescent-phase group).

In the group of 114 sera from patients diagnosed with Lyme arthritis, sensitivities of the C6 ELISA and WCS ELISA-based 2-tier testing were 98.2% and 95.6%, respectively ($P = 0.38$). Similarly, in the 8 patients with late neuroborreliosis, the sensitivities of the C6 ELISA and of 2-tier testing were both high (100%, $P > 0.99$).

WCS ELISA-based 2-tier testing was positive on sera from only 7 patients who failed to react by the C6 ELISA, of whom 5 had erythema migrans, 1 had Lyme arthritis, and 1 had early neurologic Lyme disease. Among the 200 serum samples found to be 2-tier positive by at least a positive IgG immunoblot, 2 were negative by C6 ELISA. In contrast, among the 92 sera found to be 2-tier positive based on a

Table 4
Sensitivity by symptom category in patients with Lyme disease.

Symptom category	Number [percent] of positive/indeterminate[a] (95% CI)					P^d	P^e
	C6 ELISA	WCS ELISA	Two-tier WCS ELISA[b]	Two-tier C6 ELISA[c]	*n*		
All Lyme patient sera	427 [75.0%] (71.3–78.5)	465 [81.7%] (78.3–84.8)	293 [51.5%] (47.3–55.7)	288 [50.6%] (46.4–55.7)	569	<0.001	<0.001
All erythema migrans	268 [66.5%] (61.7–71.1)	302 [74.9%] (70.4–79.1)	142 [35.2%] (30.6–40.1)	139 [34.5%] (29.9–39.4)	403	<0.001	<0.001
Single erythema migrans	77 [57.9%] (49.0–66.4)	91 [68.4%] (59.8–76.2)	36 [27.1%] (19.7–35.5)	36 [27.1%] (19.7–35.5)	133	<0.001	<0.001
Multiple erythema migrans	52 [89.7%] (78.8–96.1)	55 [94.8%] (85.6–98.9)	37 [63.8%] (50.1–76.0)	36 [62.1%] (48.4–74.5)	58	<0.001	<0.001
Acute erythema migrans	191 [64.1%] (58.4–69.5)	214 [71.8%] (66.3–76.8)	114 [38.3%] (32.7–44.0)	112 [37.6%] (32.1–43.4)	298	<0.001	<0.001
Convalescent erythema migrans	77 [73.3%] (63.8–81.5)	88 [83.8%] (75.3–90.3)	28 [26.7%] (18.5–36.2)	27 [25.7%] (17.7–35.2)	105	<0.001	<0.001
All early neurologic Lyme disease	39 [88.6%] (75.4–96.2)	43 [97.7%] (88.0–99.9)	34 [77.3%] (62.2–88.5)	33 [75.0%] (59.7–86.8)	44	0.13	0.03
Early neurologic Lyme disease—acute	18 [90.0%] (68.3–98.8)	19 [95.0%] (75.1–99.9)	16 [80.0%] (56.3–94.3)	16 [80.0%] (56.3–94.3)	20	0.5	0.5
Early neurologic Lyme disease—convalescent	21 [87.5%] (67.6–97.3)	24 [100%] (85.8–100)	18 [75.0%] (53.3–90.2)	17 [70.8%] (48.9–87.4)	24	0.38	0.13
Late neurologic Lyme disease	8 [100%] (63.1–100)	8 [100%] (63.1–100)	8 [100%] (63.1–100)	8 [100%] (63.1–100)	8	>0.99	>0.99
Lyme arthritis	112 [98.2%] (93.8–99.8)	112 [98.2%] (93.8–99.8)	109 [95.6%] (90.1–98.6)	108 [94.7%] (88.9–98.0)	114	0.38	0.13

[a] Indeterminate category refers only to ELISA results, which can be reported as positive, negative, or indeterminate; 2-tier testing is reported as either positive or negative. For purposes of calculation, the % positive values in the table in the ELISA categories sum both the positive and indeterminate results. % Positive values were calculated as total no. of positive (or positive + indeterminate) samples/total no. of samples. C6 ELISA and WCS ELISA had totals of 5 and 17 indeterminate results, respectively.

[b] Two-tier testing with WCS ELISA as the first step and IgG + IgM Western blot as the second step; for 2-tier testing, 319 serum samples were tested by Wampole WCS ELISA and 250 samples by Vidas WCS ELISA.

[c] Two-tier testing with C6 ELISA as the first step and IgG + IgM Western blot as the second step.

[d] *P* calculated for C6 ELISA versus WCS ELISA–based 2-tier testing.

[e] *P* calculated for C6 ELISA versus C6 ELISA–based 2-tier testing.

positive WCS ELISA and only a positive IgM immunoblot, 5 were negative by C6 ELISA testing (2/200 versus 5/92, $P = 0.03$).

The sensitivity of the C6 ELISA-based 2-tier testing was indistinguishable from that of WCS ELISA-based 2-tier testing overall (50.6% versus 51.5%, $P = 0.81$) and in each of the individual patient groups (Table 4).

4. Discussion

In this evaluation of the performance of serologic methods for detection of antibodies to *B. burgdorferi*, the C6 ELISA as a standalone test had significantly greater sensitivity than either the WCS ELISA-based 2-tier testing method (66.5% versus 35.2%, $P < 0.001$) or the C6 ELISA-based 2-tier testing method (66.5% versus 34.5%, $P < 0.001$) for patients with erythema migrans (Table 4). In contrast, on sera from the groups of patients with neurologic or rheumatologic manifestations of Lyme disease, each of the 3 testing methods had high sensitivity without a statistically significant difference ($P > 0.05$) (Table 4). The specificity of either of the 2-tier testing methods on more than 2200 control sera, however, exceeded that of the C6 ELISA alone by 0.6 percentage points (99.5% versus 98.9%, 95% CI surrounding the difference of 0.04 to 1.15), and this difference was significant ($P < 0.05$). The 0.4–0.7 percentage point lower specificity values observed for these serologic methods in the endemic compared with the nonendemic blood donor group might be explained by the inclusion of individuals with prior exposure to *B. burgdorferi* in the endemic group, although this hypothesis cannot be evaluated due to the lack of information on donor histories.

Our overall results for specificity are quite comparable to that for the C6 ELISA in another published study that involved a sizeable but smaller number of control serum samples (present study 98.9% [2216/2241] versus 98.4% [1279/1300], $P = 0.22$) (Branda et al., 2011). The authors of that report (Branda et al., 2011) observed that a novel 2-tier strategy using a WCS ELISA followed by the C6 ELISA would have increased the specificity to 99.5%, a figure that was identical to the conventional WCS ELISA–immunoblot-based 2-tier testing. A reanalysis of our study data with such an algorithm yielded the same 99.5% specificity value. Interestingly, however, in the group of individuals with other disease conditions as well as in the group of LymeRx® vaccine recipients, which collectively might be more representative of the target population for testing than blood donors, the specificity of this 2-tier ELISA algorithm was identical to that of the C6 ELISA alone. Serial 2-step algorithms generically offer improved specificity at the cost of some loss of sensitivity; in the present study, the serial ELISA algorithm would result in a loss of about 6% in sensitivity for erythema migrans patients (Table 4), and a similar decrease was observed by Branda et al. (2011). A cost–benefit analysis of this tradeoff may indicate its utility relative to other algorithms.

Five C6 ELISA–positive blood donor sera out of 8 that were retested 2 years later were reproducibly C6 ELISA-positive, suggesting that random assay error was not responsible for the positive results among control sera in most cases. In parallel, a subset of 188 endemic blood donor sera that were negative when first assayed—about 14% of the total in this group—were retested and yielded an almost identical distribution of absorbance readings (none positive, none indeterminate, median Lyme index value = 0.182 versus 0.155 when originally tested [Lyme index value of cut-off = 1 for reference]). These results indicate the relatively consistent performance of the C6 ELISA in testing control populations.

The low sensitivity of WCS ELISA-based 2-tier testing in detection of patients with early Lyme disease associated with erythema migrans reported in previous studies (Bacon et al., 2003; Grodzicki and Steere, 1988; Nowakowski et al., 2001) was confirmed in the present study. Sensitivity was under 30% in patients with a single erythema migrans skin lesion. By comparison, sensitivity was over twice this value when the same sera were tested by the C6 ELISA. The maximum sensitivity that can be achieved at this stage of the infection may be limited by the lag time intrinsic to the primary immune response, rather than by the specific assay methodology. In patients with multiple erythema migrans, sensitivity rose substantially with both methods, to 63.8% and 89.7% for 2-tier testing and C6 ELISA, respectively. Our results suggest that an immune response to the C6 peptide appears at an early point in time, often developing more rapidly than either the IgM or IgG response to the combination of antigens on which the 2-tier immunoblot criteria are based (Bacon et al., 2003). The *vlsE* gene encoding the C6 peptide is expressed early in the infection of mammals, but is poorly expressed in cultured borreliae, the source of antigens for WCS ELISAs and immunoblots (Crother et al., 2003).

Although some of the sera obtained from patients with erythema migrans that were evaluated in this study were specifically selected based on negative archival results with a WCS ELISA, negative results on the first-tier WCS ELISAs used in this study were not the explanation for the lower sensitivity of 2-tier testing (Table 4). The selection of the MarDx/Trinity Biotech immunoblot kits as the second tier test, however, may partially explain the reduced sensitivity, as suggested in at least 1 study of IgM immunoblot kit performance (Mogilyansky et al., 2004). An alternative explanation for the lower sensitivity of the IgM immunoblot is a reduction of IgM activity due to prolonged sample storage or a prior freeze–thaw cycle (Jobe et al., 2008; Petrakis, 1985). Against the latter hypothesis are the results of a recent report of 76 culture-positive patients with erythema migrans for whom 2-tier testing was performed prospectively using the same immunoblot kit as was used in our study (Steere et al., 2008). In that report, the 2-tier seroreactivity rate on acute-phase sera was 30.3% (23/76), which was even lower than what we had observed (38.3%).

In the current study, not all of the sera from patients with Lyme arthritis were seropositive by 2-tier testing; this unexpected result is likely attributable either to misclassification of some samples or to sample degradation as many of these samples were originally collected more than a decade earlier.

The sensitivity of the C6 ELISA was comparable to that of WCS ELISA–based 2-tier testing at 90.0% and 80.0%, respectively ($P = 0.50$), in the group of 20 acute-phase sera from patients with early neurologic Lyme disease. The sensitivity of 2-tier testing would not have changed in this group if IgM seropositivity were not restricted to an illness of ≤30 days' duration (CDC, 1995). Sera from patients with facial palsy accounted for the majority of both the acute (13/20)- and convalescent (13/24)-phase samples in the neurologic Lyme disease group. In selecting specimens from patients with extracutaneous manifestations of Lyme disease, sample selection bias may have inadvertently been introduced through the prior use of serology to characterize such specimens by the sources. Although the sensitivity of an in-house C6 ELISA in early neurologic Lyme disease patients was 60% in 1 study from the United States (Bacon et al., 2003), rates of at least 80% have been generally found in studies from both the United States (Liang et al., 1999b; Peltomaa et al., 2004; Steere et al., 2008) and Europe (Skarpaas et al., 2007; Tjernberg et al., 2008) (see below).

Several recent reports have described the performance evaluations of independently developed in-house C6 ELISA tests (Bacon et al., 2003; Branda et al., 2010; Gottner et al., 2004). The sensitivities and specificities observed in these assays have varied from those determined in the present study for the commercial C6 ELISA kit. Sensitivities and/or specificities lower than those demonstrated with the commercial kit currently marketed in the United States may be due to several factors, including different assay chemistry, reagents, or procedures, and, in some cases, use of C6 peptide sequences other than the B31 sequence used in the kit. The present study also made use of significantly larger patient and control populations than those evaluated using in-house kits in previous reports, providing a more robust statistical basis for the findings reported herein. Conclusions relating to the performance of in-house C6 ELISA assays cannot be

reliably extended to that of the commercial C6 ELISA kit evaluated in this study and vice versa.

Substitution of the C6 ELISA result for the WCS ELISA in the first tier of the 2-tier procedure yielded virtually identical results for both sensitivity and specificity, consistent with the observation that sensitivity of 2-tier testing is limited by the immunoblot step. Use of the C6 ELISA as the first-tier test, however, was associated with 82 fewer false-positive results among the 2208 (nonvaccinee) control sera, which translates to a 77.4% reduction in unnecessary immunoblot testing. These results should stimulate further cost-effectiveness studies comparing WCS ELISA–based 2-tier testing with C6 ELISA-based 2-tier testing.

Overall, our results indicate that the C6 ELISA should be preferred over 2-tier testing in patients with early infection, for example, in patients with a skin lesion(s) for whom a clinical diagnosis is uncertain. Given the ease of performance, objectivity and greater simplicity of single-tier testing using an ELISA, the C6 ELISA may be preferred over 2-tier testing in patients with early neurologic Lyme disease and other manifestations of early disseminated Lyme disease. An advantage of 2-tier testing over the C6 ELISA is its higher specificity. The high specificity of 2-tier testing found in this study, however, is not consistent with observations in clinical practice where overreading of weak bands on the IgM immunoblot in particular has served to reduce specificity and stimulate interest in alternative testing strategies to avoid the IgM immunoblot entirely (Branda et al., 2010; Seriburi et al., 2011; Weinstein, 2008). Another potential advantage of 2-tier testing over C6 ELISA is that the former provides information on the presence of an expanded IgG response specifically, a serologic prerequisite for the diagnosis of late Lyme disease (CDC, 1995; Wormser et al., 2006). Thus, for the diagnosis of late Lyme disease, it may be advantageous to perform an IgG immunoblot to supplement the C6 or another ELISA.

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